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Fungi are ever changing their secondary metabolites profile in order to adapt to their environment. Previously characterized fungal cultures were re-analyzed with the intention of re-isolating scaled up quantities of metabolites of interest. However, in doing so there were several instances where fungal secondary metabolites were isolated that were not previously observed. The biological activities of the isolated secondary metabolites were evaluated using a variety of bioassays. Three compounds that were not previously isolated were characterized and added to our compound library. Re-isolation and re-analysis of fungal secondary metabolites can lead to the discovery of unsought yet interesting changes in secondary metabolite profile and biological activity.

To take advantage of fungi's ability to adapt to their environment, our lab has grown fungi in co-culturing experiments in hopes of activating silent biosynthetic gene clusters thus diversifying secondary metabolite production. The most common fungus utilized in these experiments has been *Xylaria flabelliformis* (formerly known as *Xylaria cubensis*) due to its production of the fungistatic compound and FDA approved drug, griseofulvin. In this study, two other *Xylaria* spp. are analyzed in comparison to *Xylaria flabelliformis* to determine variation in griseofulvin production among the strains and over time. Characterizing griseofulvin production by various *Xylaria* spp. leads to intentionality in future co-culturing experimental design, in that the optimal *Xylaria* sp. can be selected.

THE PURSUIT OF CHEMICAL DIVERSITY IN FUNGI

by

Allison Jade Wright

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Approved by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

1.1 Re-Isolation of Fungal Secondary Metabolites

Fungi have the ability to biosynthesize a variety of secondary metabolites, such as mycotoxins, pigments, and antimicrobials. Re-isolation allows us to re-analyze previously characterized secondary metabolite profiles. In this re-analysis new analogues and different biological activities can be discovered. Re-isolation and re-analysis of fungi should be done to isolate the previously characterized secondary metabolites as well as the newly present compounds because they are potentially advantageous.[1] Despite compound library curation, many previously isolated compounds are not available for new bioassay testing.

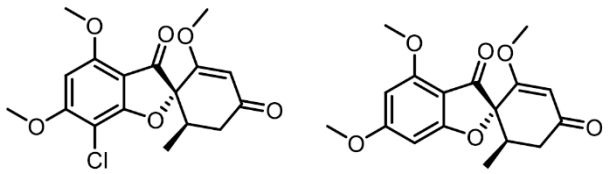
1.2 Co-Culturing

Fungi have the extraordinary ability to survive in an assortment of environments, including soil, plants and human tissues.[2] They are able to survive in these environments by overcoming competition in various ways, such as sporulation, stress recovery, rapid growth and the use and nullification of inhibitors.[3] It has been shown that fungi are only producing a fraction of their potential secondary metabolites when grown under standard laboratory conditions.[4-6] A variety of biosynthetic gene clusters, that are not associated with known compounds, have been discovered and classified as silent biosynthetic gene

clusters. Co-culturing is a means to taking advantage of competitive traits by provoking a stress response which could in turn activate silent biosynthetic gene clusters. Thus, co-culturing has the potential to diversify secondary metabolite biosynthesis in fungi. Changes in the secondary metabolite profile due to co-culturing can be monitored *in situ* via the droplet-liquid microjunction-surface sampling probe (droplet probe)[7].

1.3 Secondary Metabolites Produced by *Xylaria* spp.

Griseofulvin (Figure 1a) was first discovered in 1939 in London, and today is commonly used for its antifungal properties as an FDA-approved drug.[8-11] Since then, it has been determined that *Xylaria* sp. predominantly produce the secondary metabolites griseofulvin and dechlorogriseofulvin (Figure 1b). For example, *Xylaria flabelliformis* (previously known as *Xylaria cubensis*) is commonly found in nature as an endophyte (endosymbiotic) and decomposer (saprobic) and biosynthesizes the secondary metabolites griseofulvin and dechlorogriseofulvin.[12] The production of griseofulvin has helped *Xylaria* sp. survive and thrive in various environments because of griseofulvin's fungistatic properties, meaning that it inhibits fungal growth rather than killing competing fungi.[4]



(a) Griseofulvin (b) Dechlorogriseofulvin

Figure 1. (a) Griseofulvin Structure with Absolute Stereochemistry (b) Dechlorogriseofulvin Structure with Absolute Stereochemistry

Griseofulvin's fungistatic properties have led to various co-culturing studies based on the hypothesis that by inducing stress on another fungus the secondary metabolite production would change due to the two species interacting.[4, 6] Fungi are biological organisms and present an array of biological variance both morphologically and chemically. In this study, the varying production of griseofulvin and dechlorogriseofulvin is related to the morphological variation, which can be used in future studies to design co-culturing experiments.

CHAPTER II

RE-ISOLATION OF FUNGAL SECONDARY METABOLITES

2.1 Experimental

2.1.1 Fungal Culture Production

G-strain fungal cultures are maintained on Potato Dextrose Agar (PDA; Difco) media and transferred periodically. To begin a new fungal culture, an agar plug with mycelium is transferred to Yeast Extract Soy Peptone Dextrose (YESD) broth. This seed culture is then incubated for three days at room temperature with agitation (~100 rpm). Then, the seed culture is transferred to 250 mL Erlenmeyer flasks containing 10 g of autoclaved rice and left to grow at room temperature for two weeks.

2.1.2 Extraction and Purification

A 1:1 methanol:chloroform mixture was added to the fermented fungal cultures, chopped, and shaken (~100 rpm) for at least 4 hours at room temperature. The culture was then vacuum filtered, and to the filtrate 1:2 chloroform:water was added. The filtrate was stirred and transferred to separatory funnel where the bottom layer (chloroform) was drawn off and evaporated to dryness under vacuum. The now de-sugared extract was reconstituted in 1:1:2 acetonitrile:methanol:hexane to remove the fats. The resulting mixture was then mixed vigorously and transferred to a separatory funnel where the bottom layer (1:1 acetonitrile:methanol) was collected and evaporated to dryness under

vacuum. The extract was then subjected to an initial chromatographic separation via normal phase flash chromatography on a Teledyne ISCO Combiflash Rf 200 which was monitored by an evaporative light scattering detector (ELSD) and photodiode array (PDA) detector. The semi-purified fractions from flash chromatography are then subjected to further purification via reverse HPLC. The HPLC separations were performed with an Atlantis T3 C_{18} preparative (5 µm; 19 mm × 250 mm) and semi-preparative (5 µm; 10 mm × 250 mm) columns, at a flow rate of 16.9 ml/min and 4.6 ml/min, respectively, with a Varian Prostar HPLC system equipped with a Prostar 210 pump and a Prostar 335 PDA detector, with the collection and analysis of data using Galaxie Chromatography Workstation software.

2.1.3 Identification and Characterization of Fungal Secondary Metabolites

The structures of the secondary metabolites were identified via a suite of spectroscopic and spectrometric techniques. The HRESIMS data were collected on either a Thermo LTQ Orbitrap XL mass spectrometer or a Thermo Q Exactive Plus (Thermo Fisher Scientific); both equipped with an electrospray ionization source and a Waters Acquity UPLC (Waters Corp.) using a BEH C_{18} column (1.7 µm; 50 mm × 2.1 mm) set to a temperature of 40ºC and a flow rate of 0.3 ml/min. The mobile phase consisted of a linear gradient of CH_3CN-H_2O (both acidified with 0.1% formic acid), starting at 15% CH_3CN and increasing linearly to 100% CH_3CN over 8 minutes, with a 1.5-minute hold before returning to the starting conditions. The NMR data were collected using a JOEL ECS-400 spectrometer equipped with a JOEL normal geometry broadband Royal probe and 24-slot autosampler which was operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C as

well as a JEOL ECA-500 spectrometer which was operated at 500 MHz for ^{1}H and 125 MHz for ^{13}C (Both from JEOL USA, Inc.).

2.2 Results

Fifteen fungal strains were grown for re-isolation (Figure 2). Among these fungi, there were 92 secondary metabolites that were expected to be isolated based on secondary metabolites previously isolated from these fungal strains by our laboratory (Table 1). Of those 92, 19 were isolated and identified. Additionally, three compounds that our lab had not previously isolated were isolated and identified. In total, 22 compounds were isolated via re-isolation (Figure 4).

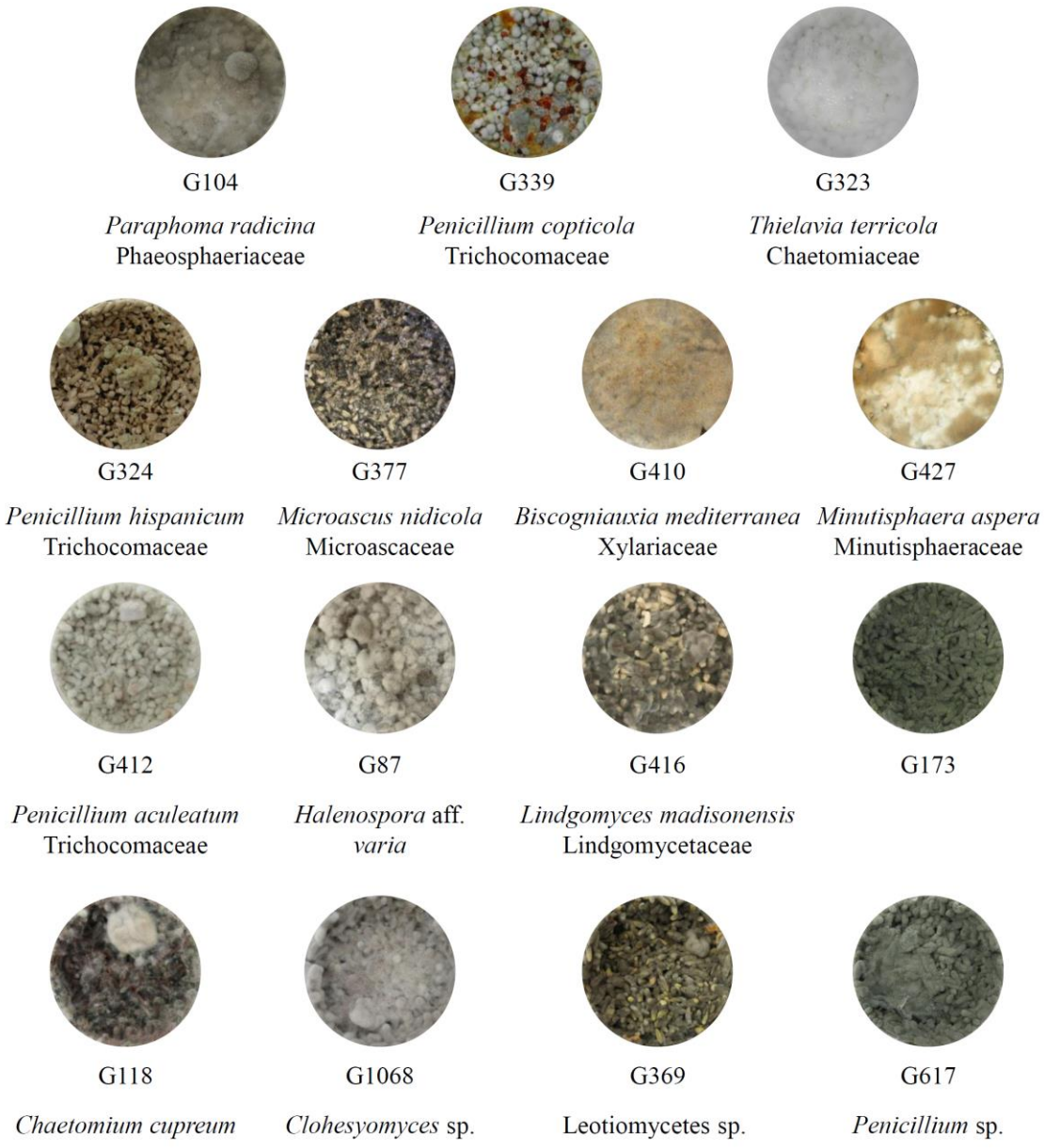
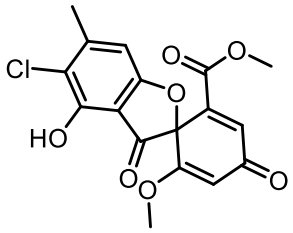
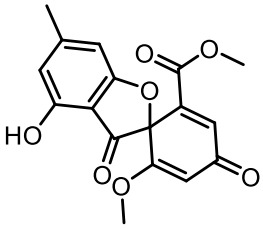
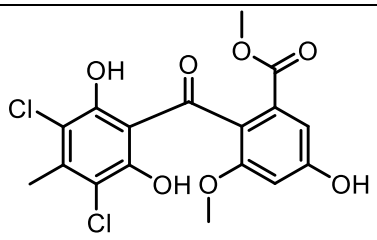
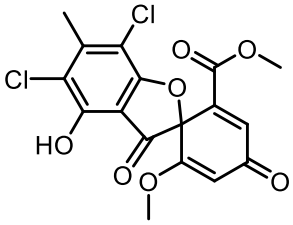
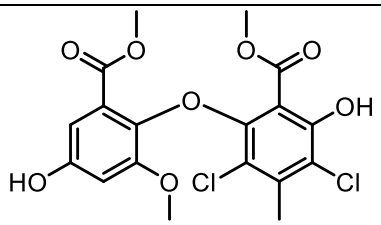
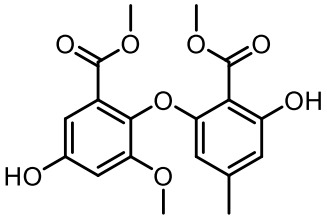
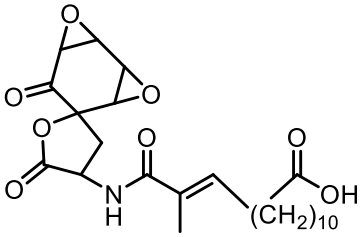
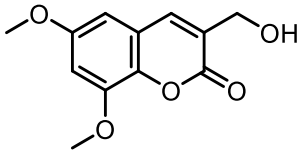
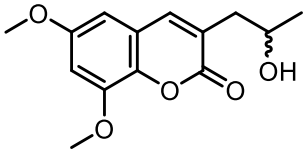
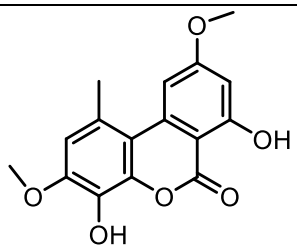
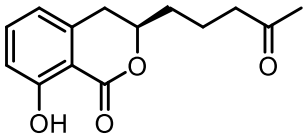
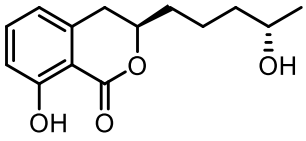
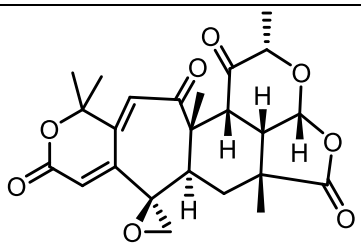
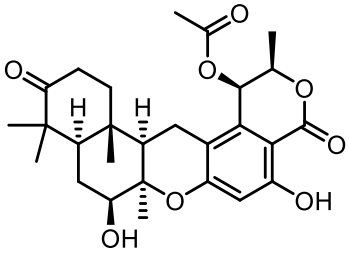


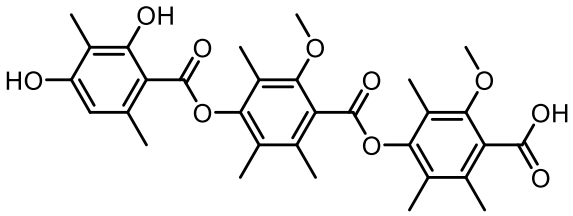
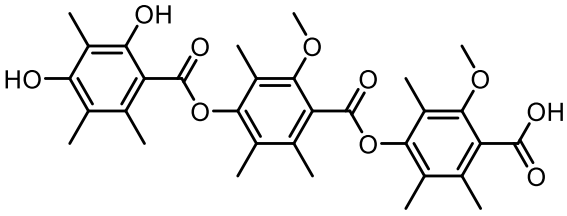
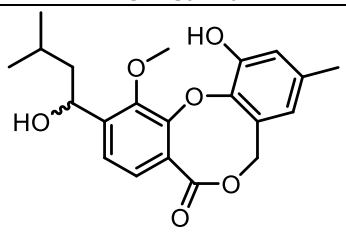
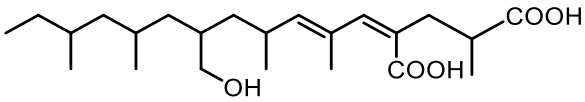
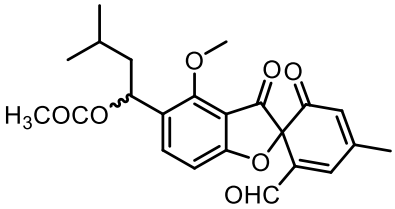
Figure 2. Fungal Cultures (Top View) Grown on Solid Rice Media

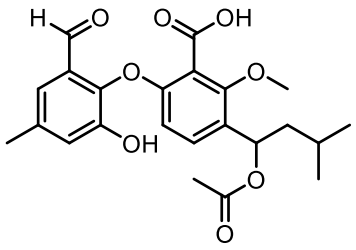
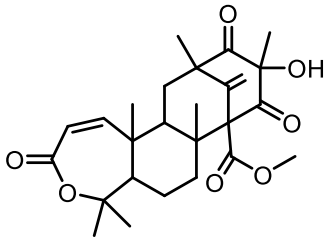
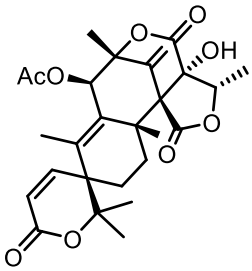
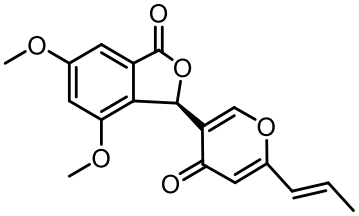
Table 1. The Secondary Metabolites Pursued and Isolated with Amounts Prior to and Following Re-Isolation.

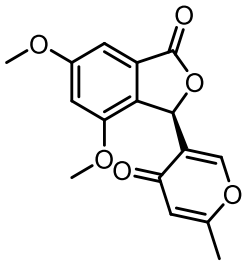
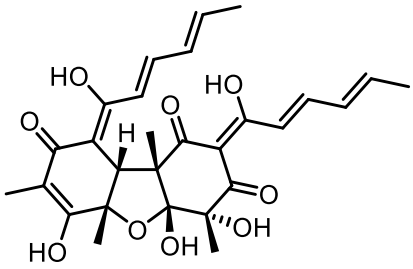
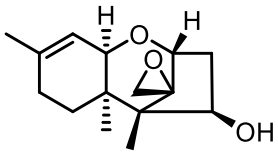
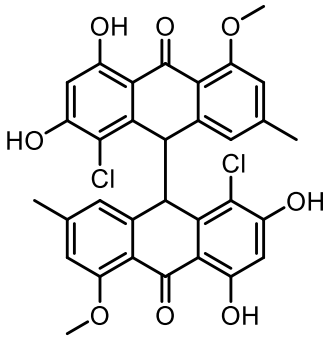
#	Strain	Compound	Amount before	Amount after
1	G77	 Antibiotic SS 19508D; SS 19508D $C_{17}H_{13}O_7Cl$	2.79 mg	2.79 mg
2	G77	 Bisdechlorogeodin $C_{17}H_{14}O_7$	2.28 mg	2.28 mg
3	G77	 Dihydrogeodin $C_{17}H_{14}O_7Cl_2$	1.27 mg	1.27 mg
4	G77	 Geodin $C_{17}H_{12}O_7Cl_2$	12.24 mg	12.24 mg

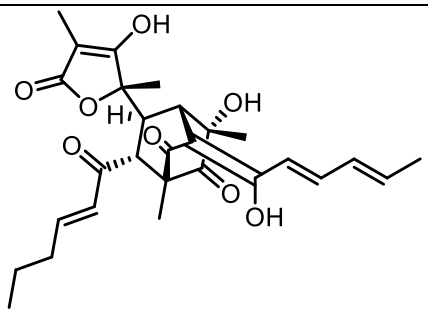
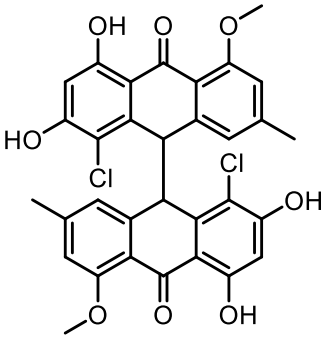
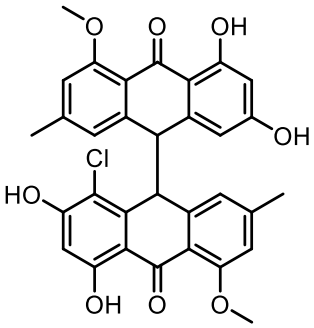
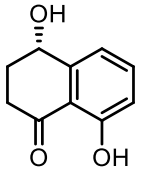
5	G77	 Methyl 2,4-dichloroasterrate $C_{18}H_{16}O_8Cl_2$	2.39 mg	2.39 mg
6	G77	 Methyl asterrate; Trimethylosoic acid $C_{18}H_{18}O_8$	4.18 mg	4.18 mg
7	G118	 Chaetocuprum $C_{24}H_{33}NO_8$	2.96 mg	2.96 mg
8	G173	 3-Hydroxymethyl-6,8-dimethoxycoumarin $C_{12}H_{12}O_5$	7.23 mg	8.50 mg
9	G173	 Pestalasin A $C_{14}H_{16}O_5$	0 mg	0 mg

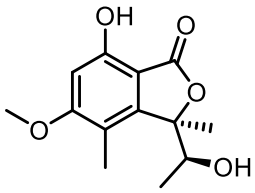
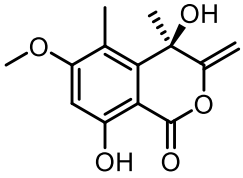
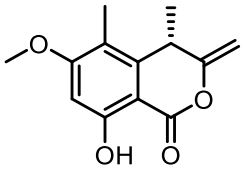
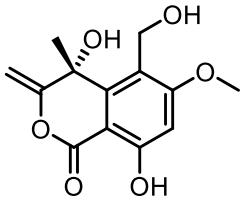
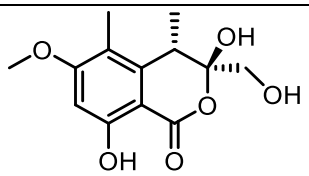
10	G173	 Graphislactone A $C_{16}H_{14}O_6$	1.25 mg	0 mg
11	G173	 Aspergillumarin A $C_{14}H_{16}O_4$	3.22 mg	3.22 mg
12	G173	 Aspergillumarin B $C_{14}H_{18}O_4$	0 mg	1.75 mg
13	G173	 Berkeleyacetal C $C_{24}H_{26}O_8$	3.81 mg	10.97 mg
14	G173	 Thailandolide B $C_{27}H_{34}O_8$	0.23 mg	0.23 mg

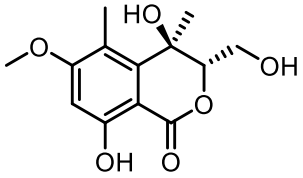
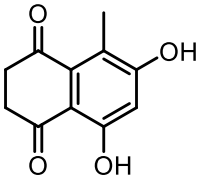
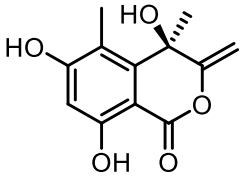
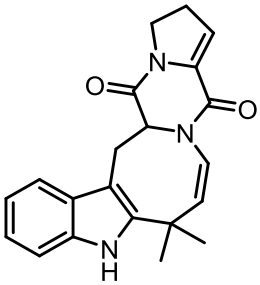
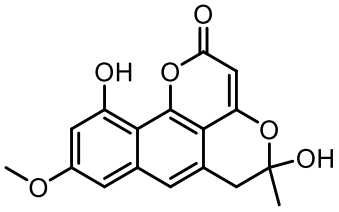
15	G323	 Thielavin B $C_{31}H_{34}O_{10}$	0 mg	53.27 mg
16	G323	 Thielavin C $C_{32}H_{36}O_{10}$	3.55 mg	3.55 mg
17	G412	 Paeciloxocin A $C_{21}H_{24}O_6$	5.06 mg	5.06 mg
18	G412	 Radiclonic acid $C_{23}H_{40}O_5$	0.34 mg	11.71 mg
19	G412	 Purpactin C $C_{23}H_{24}O_7$	6.13 mg	6.13 mg

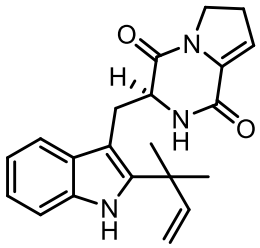
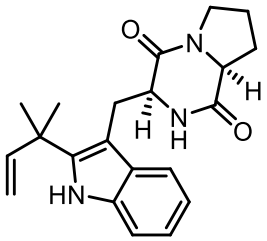
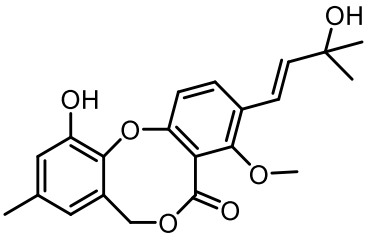
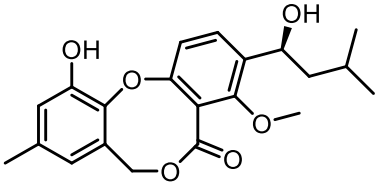
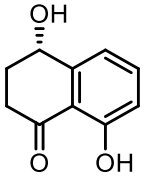
20	G412	 Tenellic acid C $C_{23}H_{26}O_7$	1.00 mg	1.00 mg
21	G617	 Preaustinoid A 2 $C_{26}H_{34}O_7$	*	1.24 mg
22	G617	 Austin $C_{27}H_{32}O_9$	*	21.30 mg
23	G617	 Vermistatin $C_{18}H_{16}O_6$	6.50 mg	6.50 mg

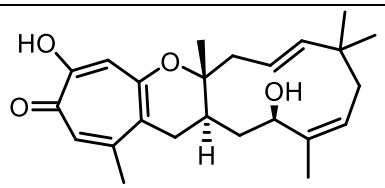
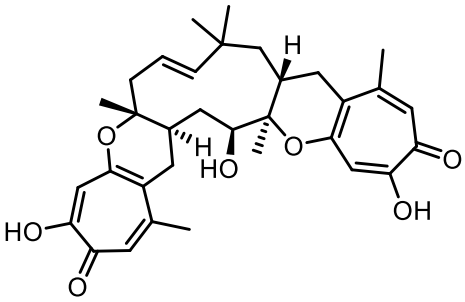
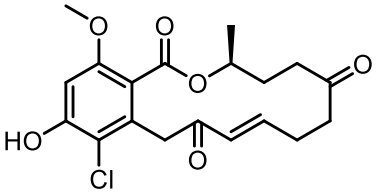
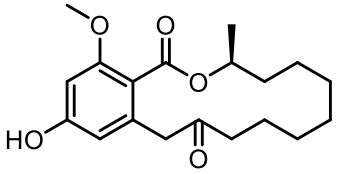
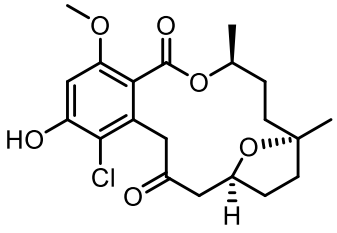
24	G617	 Penisimplicissin $C_{16}H_{14}O_6$	0.49 mg	0.49 mg
25	G339	 Bisvertinolone $C_{28}H_{32}O_9$	6.26 mg	6.26 mg
26	G339	 Trichodermol $C_{15}H_{22}O_3$	0 mg	0 mg
27	G339	 Neobulgarone F $C_{32}H_{24}Cl_2O_8$	3.51 mg	3.51 mg

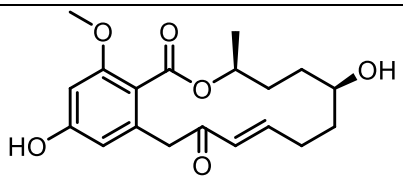
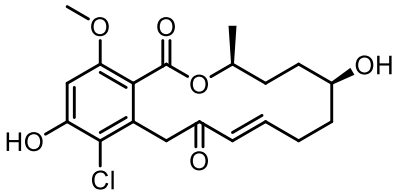
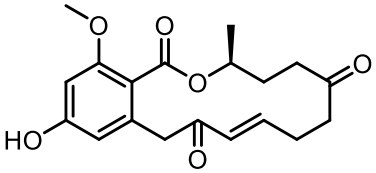
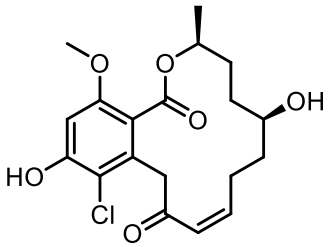
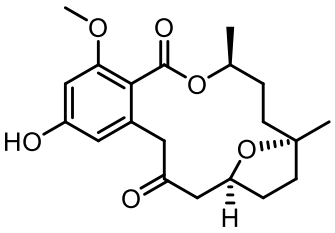
28	G339	 Bisorbibutenolide $C_{28}H_{32}O_8$	2.61 mg	2.61 mg
29	G339	 Neobulgarone E $C_{32}H_{24}Cl_2O_8$	3.96 mg	3.96 mg
30	G339	 Neobulgarone C $C_{32}H_{25}ClO_8$	1.24 mg	1.24 mg
31	G104	 Isosclerone $C_{10}H_{10}O_3$	0 mg	0 mg

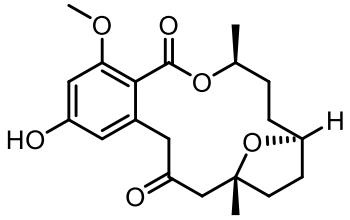
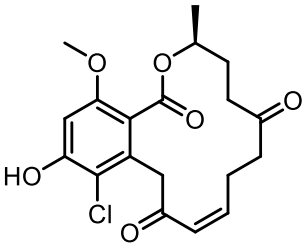
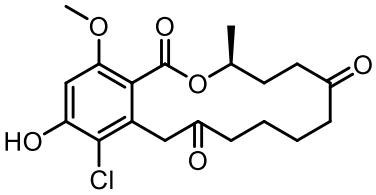
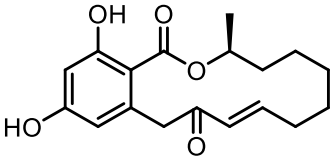
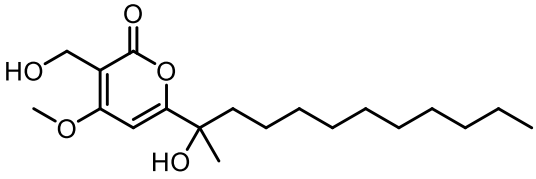
32	G104	 (*R*)-7-Hydroxy-3-((*S*)-1-hydroxyethyl)-5-methoxy-3,4-dimethylisobenzofuran- 1(3H)-one $C_{13}H_{16}O_5$	2.10 mg	2.10 mg
33	G104	 (*R*)-3,4-Dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl- 3-methyleneisochromen-1-one $C_{13}H_{14}O_5$	27.76 mg	33.87 mg
34	G104	 Clearanol C $C_{13}H_{14}O_4$	2.19 mg	2.19 mg
35	G104	 Clearanol G $C_{13}H_{14}O_6$	0.27 mg	0.27 mg
36	G104	 (3*R*,4*S*)-3,8-Dihydroxy-3-hydroxymethyl-6-methoxy-4,5-dimethyl-isochroman-1-one $C_{13}H_{16}O_6$	3.39 mg	3.39 mg

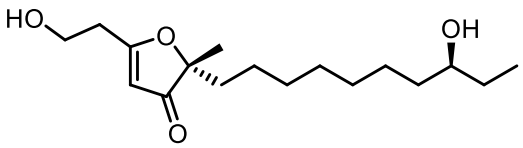
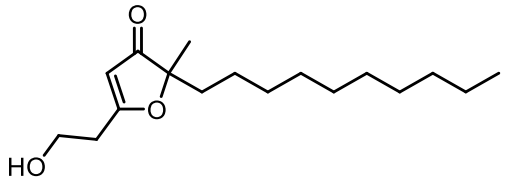
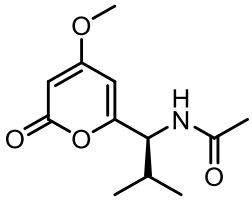
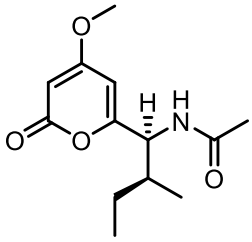
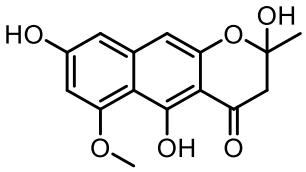
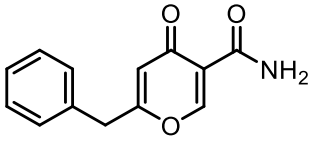
37	G104	 Clearanol F $C_{13}H_{16}O_6$	0.37 mg	0.37 mg
38	G104	 Radinaphthalenone $C_{11}H_{10}O_4$	0.38 mg	0.38 mg
39	G104	 (4*R*)-3,4-Dihydro-4,6,8-trihydroxy-4,5-dimethyl-3-methyleneisochromen-1-one $C_{12}H_{12}O_5$	2.92 mg	2.92 mg
40	G324	 10,20-Dehydro[12,13-dehydroprolyl-2-(1,1-dimethylallyl)tryptophyl]diketopiperazine] $C_{21}H_{21}N_3O_2$	1.93 mg	1.93 mg
41	G324	 Pannorin B $C_{17}H_{14}O_6$	4.25 mg	4.25 mg

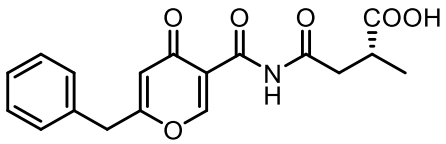
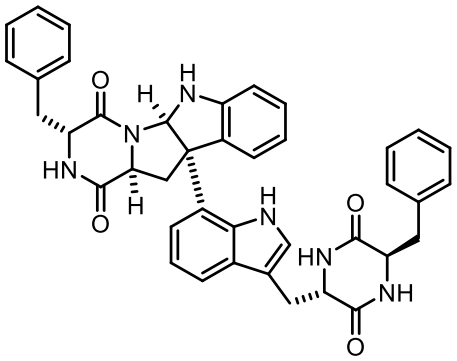
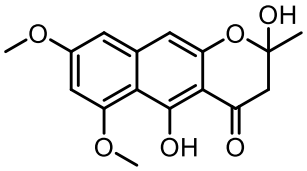
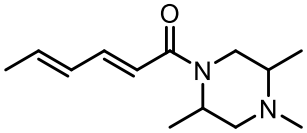
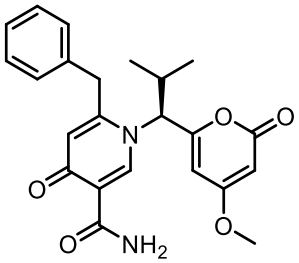
42	G324	 12,13-Dehydroprolyl-2-(1,1-dimethylallyltryptophyl)diketopiperazine $C_{21}H_{23}N_3O_2$	0.56 mg	0.56 mg
43	G324	 Deoxybrevianamide E $C_{21}H_{25}N_3O_2$	0.32 mg	0.32 mg
44	G369	 Dehydroisopenicillide $C_{21}H_{22}O_6$	*	0.88 mg
45	G369	 Penicillide; Vermixocin A $C_{21}H_{24}O_6$	3.11 mg	14.32 mg
46	G369	 Isosclerone $C_{10}H_{10}O_3$	0 mg	0 mg

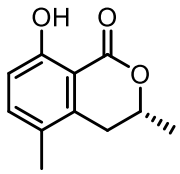
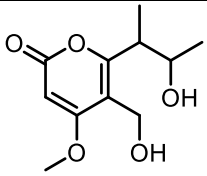
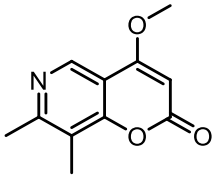
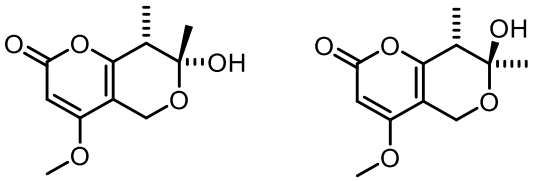
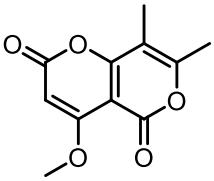
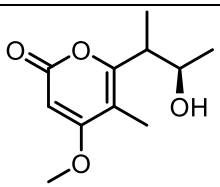
47	G369	 Epolone B $C_{24}H_{32}O_4$	2.76 mg	2.76 mg
48	G369	 Pycnidione $C_{33}H_{40}O_7$	1.61 mg	1.61 mg
49	G87	 Greensporone A $C_{19}H_{21}ClO_6$	5.33 mg	10.05 mg
50	G87	 8,9-Dihydrogreensporone C $C_{19}H_{26}O_5$	3.89 mg	3.89 mg
51	G87	 Greensporone F $C_{19}H_{23}ClO_6$	0.40 mg	0.40 mg

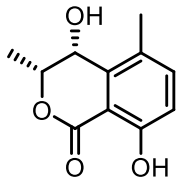
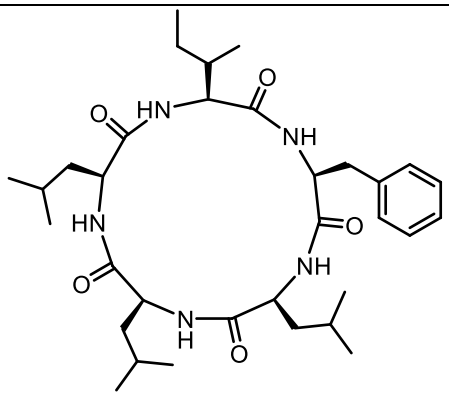
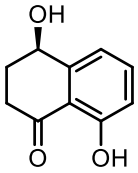
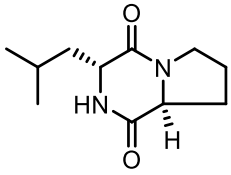
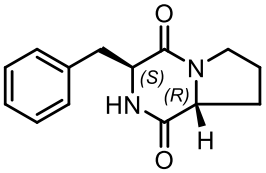
52	G87	 Dechlorogreensporone D $C_{19}H_{24}O_6$	0.72 mg	0.72 mg
53	G87	 Greensporone D $C_{19}H_{23}ClO_6$	0.28 mg	0.28 mg
54	G87	 Dechlorogreensporone A $C_{19}H_{20}O_6$	0 mg	0 mg
55	G87	 Greensporone E $C_{19}H_{23}ClO_6$	0 mg	0 mg
56	G87	 Dechlorogreensporone F $C_{19}H_{22}O_6$	0 mg	0 mg

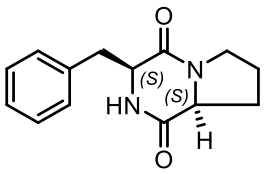
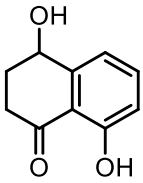
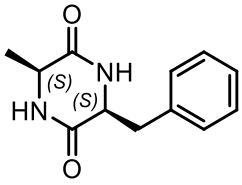
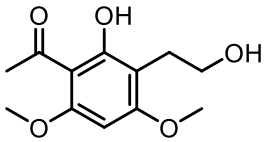
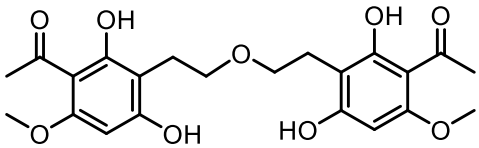
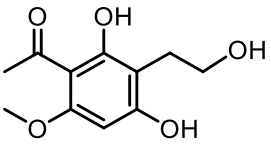
57	G87	 Greensporone G $C_{19}H_{24}O_6$	0 mg	0 mg
58	G87	 Greensporone B $C_{19}H_{21}ClO_6$	0.10 mg	1.79 mg
59	G87	 8,9-Dihydrogreensporone A $C_{19}H_{23}ClO_6$	0.30 mg	0.87 mg
60	G87	 O-Desmethylgreensporone C $C_{18}H_{22}O_5$	0.26 mg	0.26 mg
61	G377	 Monascuspyrone $C_{19}H_{32}O_5$	0 mg	1.30 mg

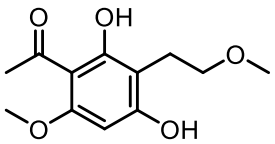
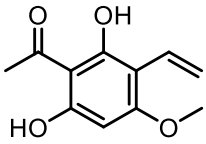
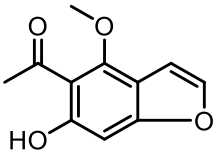
62	G377	 Monascuskaoliaone B $C_{17}H_{30}O_4$	0 mg	0 mg
63	G377	 Monascuskaoliaone $C_{17}H_{30}O_3$	0 mg	0.32 mg
64	G377	 Campyrone C $C_{12}H_{17}NO_4$	5.49 mg	5.49 mg
65	G377	 Campyrone A $C_{13}H_{19}NO_4$	0.70 mg	0.70 mg
66	G377	 Fonsecin $C_{15}H_{14}O_6$	0 mg	0 mg
67	G377	 Carbonarone A $C_{13}H_{11}NO_3$	1.74 mg	1.74 mg

68	G377	 *epi*-Pestalamide A $C_{18}H_{17}NO_6$	3.78 mg	3.78 mg
69	G377	 Asperazine $C_{40}H_{36}N_6O_4$	0 mg	0 mg
70	G377	 Fonsecin B $C_{16}H_{16}O_6$	0 mg	0 mg
71	G377	 Nigragillin $C_{13}H_{22}N_2O$	0 mg	0 mg
72	G377	 Nidiascin C $C_{23}H_{24}N_2O_5$	0.54 mg	0.54 mg

73	G410	 (*R*)-5-Methylmellein $C_{11}H_{12}O_3$	0 mg	9.25 mg
74	G410	 Chlamydospordiol $C_{11}H_{16}O_5$	0 mg	0 mg
75	G410	 Acuminatopyrone $C_{11}H_{11}NO_3$	0 mg	5.42 mg
76	G410	 Chlamydosporol (Mixture of diastereomers) $C_{11}H_{14}O_5$	0 mg	0 mg
77	G410	 Biscognin B $C_{11}H_{10}O_4$	2.27 mg	3.04 mg
78	G410	 Biscognin A $C_{11}H_{16}O_4$	0.77 mg	0.77 mg

79	G410	 (3*R*,4*R*)-*cis*-4-Hydroxy-5-methylmellein $C_{11}H_{12}O_4$	0 mg	0 mg
80	G410	 Cyclo-[*L*-Phe-*L*-Leu-*L*-Leu-*L*-Leu-*L*-Ile] $C_{33}H_{53}N_5O_5$	8.59 mg	8.59 mg
81	G427	 Regiolone $C_{10}H_{10}O_3$	0.40 mg	2.75 mg
82	G427	 Cyclo-((*S*)-Pro-(*S*)-Leu) $C_{11}H_{18}N_2O_2$	0 mg	0 mg
83	G427	 Cyclo-(*R*-Pro-*S*-Phe) $C_{14}H_{16}N_2O_2$	0 mg	0 mg

84	G427	 Cyclo-(*S*-Pro-*S*-Phe) $C_{14}H_{16}N_2O_2$	0 mg	0 mg
85	G427	 Isosclerone $C_{10}H_{10}O_3$	1.58 mg	1.58 mg
86	G427	 Cyclo-(*S*-Ala-*S*-Phe) $C_{12}H_{14}N_2O_2$	0 mg	0 mg
87	G416	 4'-Methoxymadisone $C_{12}H_{16}O_5$	0 mg	1.37 mg
88	G416	 Dimadisone $C_{22}H_{26}O_9$	0 mg	0.77 mg
89	G416	 Madisone $C_{11}H_{14}O_5$	0 mg	9.20 mg

90	G416	 2'-Methoxymadisone $C_{12}H_{16}O_5$	0 mg	0 mg
91	G416	 Dehydromadisone $C_{11}H_{12}O_4$	0 mg	0 mg
92	G416	 Visnaginone $C_{11}H_{10}O_4$	0 mg	0 mg

*Secondary metabolite was not previously isolated in the Oberlies research group.
Amounts shown in green represent an increase in isolated material as part of this project.

The extracts, initial liquid chromatography fractions, and pure compounds were tested in a variety of bioassays including for anti-cancer, anti-bacterial, and anti-amoeba activities.

2.2.1 Thielavin B

Thielavin B was first isolated in 1978 in Japan.[13] In 1981, it was isolated from *Thielavia terricola* and found to have potent inhibition of prostaglandin biosynthesis. The concentration required for 50% inhibition of the conversion of 14C-arachidonic acid into prostaglandins F2 alpha plus E2 was 9 μM.[14] Thielavin B was isolated from *Thielavia terricola* (Chaetomiaceae) (G323) and tested in various biological assays. It was inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays; however, it was active against

Staphylococcus aureus with 97.7% (±0.3) inhibition at 100 μg/mL and 61.2% (±1.4) inhibition at 10 μg/mL (Figure 5).

2.2.2 Radiclonic Acid

Radiclonic acid was isolated in Japan in 1973.[15] It was found to stimulate the growth of Chinese cabbage seedling.[16] Radiclonic acid was isolated from *Penicillium aculeatum* (Trichocomaceae) (G412) and tested in various biological assays. It was inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

2.2.3 Regiolone

Regiolone was first isolated in 1974 from *Sclerotinia sclerotiorum* and found to stimulate root elongation of rice seedlings at low concentrations and inhibit growth of shoots and roots at high concentrations.[17] Regiolone was isolated from *Minutisphaera aspera* (Minutisphaeraceae) (G427) tested in various biological assays. It was inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

2.2.4 Biscognin B

Biscognin B was isolated in 2015 from milk thistle endophytes and the biological activity was not tested.[18] Biscognin B was isolated from *Biscogniauxia mediterranea* (Xylariaceae) (G410) and found to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassay.

2.2.5 5-Methylmellein

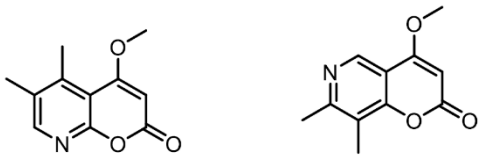
5-Methylmellein was first isolated in 1966 in Rome from *Fusicoccum amygdali*.[19] The HRMS data, ^{1}H NMR, and ^{13}C NMR were collected and compared to the reported literature values (Table 2).[20] 5-Methylmellein was isolated from *Biscogniauxia mediterranea* (Xylariaceae) (G410) and found to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

Table 2. ^{1}H NMR Data for 5-Methylmellein in $CDCl_3$

	δ_H (J, Hz)		δ_C, type	
Position	Literature[20] (300 MHz)	Observed (400 MHz)	Literature[20] (75 MHz)	Observed (100 MHz)
1	-	-	170.321	170.49
3	4.689 *m*	4.68 *ddq* (12.61; 6.31; 2.93; 2.93; 2.93)	75.405	75.57
3-CH_3	1.555 *d*	1.51 *d* (6.32)	20.877	21.07
4	2.718 *m* 2.951 dd	2.72 *dd* (16.66, 11.62) 2.94 *dd* (16.66, 3.36)	31.852	32.04
4a	-	-	137.019	137.19
5	-	-	124.894	125.05
5-CH_3	2.198 *s*	2.19 *s*	18.058	18.25
6	6.817 *d*	6.82 *d* (8.53)	115.621	115.81
7	7.291 *d*	7.29 *d* (8.53)	137.899	138.07
8	-	-	160.415	160.60
8-OH	11.005 *s*	11.00 *s*	-	-
8a	-	-	108.008	108.20

2.2.6 Acuminatopyrone

In 1991, acuminatopyrone was isolated from *Fusarium acuminatum*. However, the initial structure (Figure 3a) was reported incorrectly[21]. The revised structure (Figure 3b) of acuminatopyrone was established in 1994 by isolating the compound from two *Fusarium* spp. and characterized using NOEDIF and HETCOR NMR experiments.[22] Acuminatopyrone was isolated from *Biscogniauxia mediterranea* (Xylariaceae) (G410) and found to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.



(a) Acuminatopyrone (Original) (b) Acuminatopyrone (Revised)

Figure 3. Original and Revised Structures of Acuminatopyrone

2.2.7 (R)-3,4-Dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochromen-1-one

(*R*)-3,4-Dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochromen-1-one was first isolated in 2002 in Thailand.[23] (*R*)-3,4-Dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl-3-methyleneisochromen-1-one was isolated from *Paraphoma radicina* (Phaeosphaeriaceae) (G104) and tested in a variety of bioassays; however, it was inactive in all of them.

2.2.8 Preaustinoid A 2

Preaustinoid A 2 was first isolated in 2003 from a *Penicillium* sp. Preaustinoid A 2 was isolated from G617 which has been identified as a *Penicillium* sp. The structure was elucidated using HRMS and ^{1}H NMR data with reference to the published values (Table 3).[24] It was found to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

Table 3. ^{1}H (400 MHz) NMR Data for Preaustinoid A 2 in $CDCl_3$

Position	Literature Values δ_H (J, Hz)	Observed δ_H (J, Hz)
1	6.04 *d* (12)	6.03 *d* (11.91)
2	5.81 *d* (12)	5.80 *d* (11.86)
5	1.79 *dd* (13; 3)	1.79 *m*
6	1.49 *m* 1.70 *m*	1.51 *s* 1.70 *m*
7	1.87 *ddd* (13; 13; 3) 2.24 *ddd* (13; 3; 3)	1.86 *ddd* (12.88; 12.68; 3.63) 2.23 *ddd* (13.73; 3.17; 3.17)
9	0.65 *dd* (13; 3)	0.64 *dd* (13.36; 2.53)
11	2.0 *dd* (13; 3) 1.75 *dd* (13; 13)	1.99 *dd* (13.21; 2.45) 1.78 *dd* (9.96; 2.63)
12	1.33 *s*	1.32 *s*
13	1.18 *s*	1.16 *s*
14	1.39 *s*	1.38 *s*
15	1.42 *s*	1.41 *s*
1’	4.92 *s* 5.45 *s*	4.91 *d* (0.82) 5.44 *d* (0.82)
9’	1.52 *s*	1.51 *s*
10’	1.37 *s*	1.36 *s*
OCH_3	3.74 *s*	3.73 *s*
OH	3.28 *s*	3.23 *s*

2.2.9 Austin

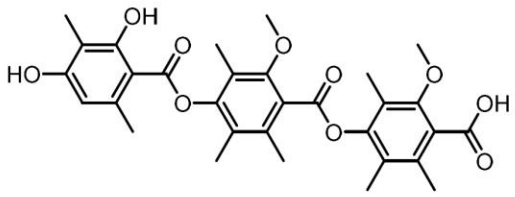
Austin was first isolated in 1976.[25] Austin was isolate from a *Penicillium* sp. (G617) and found to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

2.2.10 3-(Hydroxymethyl)-6,8-dimethoxy-2H-1-benzopyran-2-one

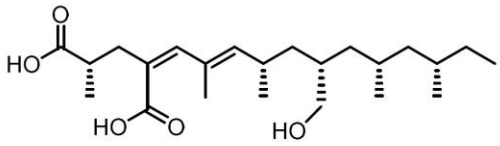
3-(Hydroxymethyl)-6,8-dimethoxy-2H-1-benzopyran-2-one was first isolated in 1990 in Canada.[26] In this project, 3-(Hydroxymethyl)-6,8-dimethoxy-2H-1-benzopyran-2-one was isolated from an unidentified freshwater fungus (G173). It was tested in anti-cancer, anti-bacterial, and anti-amoeba bioassays; however, it was found to be inactive in all of them.

2.2.11 Aspergillumarin B

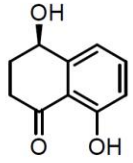
Aspergillumarin B was first isolated in 2012 in China from a marine-derived fungus, *Aspergillus* sp., which was isolated from a leaf of the mangrove tree *Bruguiera gymnorrhiza*. It was determined that Aspergillumarin B exhibits weak antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* at a concentration of 50 μg/mL.[27] Aspergillumarin B isolated from an unidentified freshwater fungus (G173). It was tested and found to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.



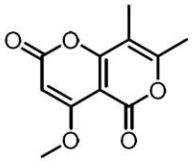
Thielavin B[13]
(G323)



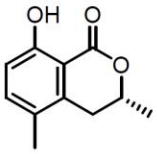
Radiclonic Acid[15]
(G412)



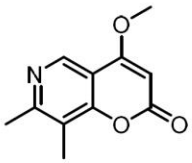
Regiolone[17]
(G427)



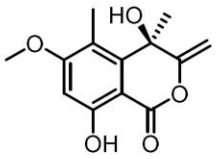
Biscognin B[18]
(G410)



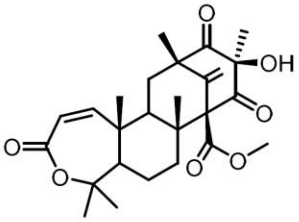
(*R*)-5-Methylmellein[19,20]
(G410)



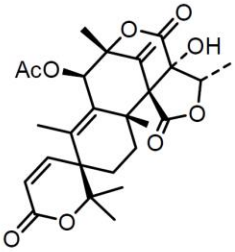
Acuminatopyrone[22]
(G410)



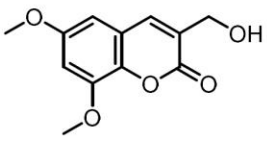
(*R*)-3,4-Dihydro-4,8-dihydroxy-6-methoxy-4,5-dimethyl3-methyleneisochromen-1-one[23]
(G104)



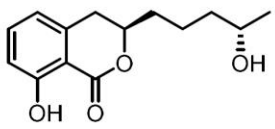
Preaustinoid A 2[24]
(G617)



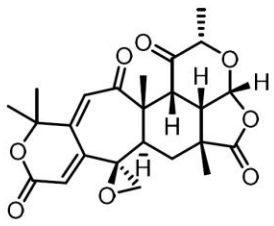
Austin[25]
(G617)



3-(Hydroxymethyl)-6,8-dimethoxy-2H-1-benzopyran-2-one[26]
(G173)

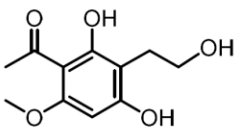


Aspergillumarin B[27]
(G173)

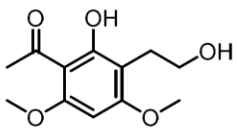


Berkeleyacetal C[28]
(G173)

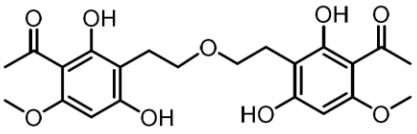
Figure 4. Secondary Metabolites Isolated and Characterized



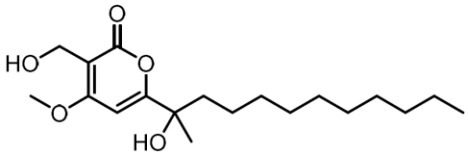
Madisone[29]
(G416)



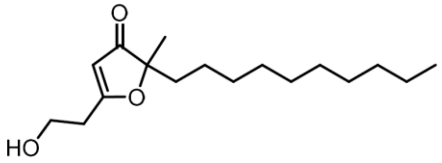
4'-Methoxymadisone[29]
(G416)



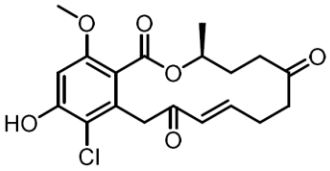
Dimadisone[29]
(G416)



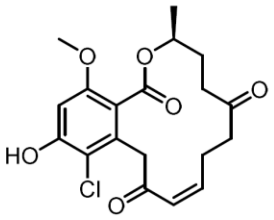
Monascuspyrone[30]
(G377)



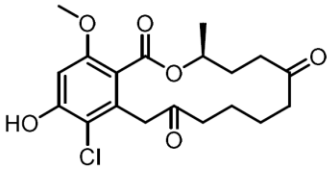
Monascuskaoliaone[31]
(G377)



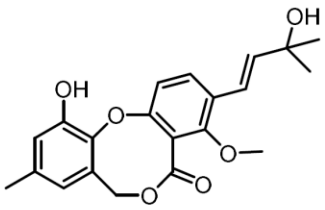
Greensporone A[32]
(G87)



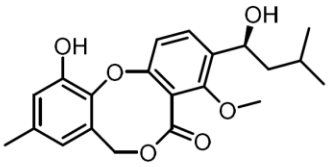
Greensporone B[32]
(G87)



8,9-Dihydrogreensporone A[32]
(G87)



Dehydroisopenicillide[33]
(G369)



Penicillide; Vermixocin A[34]
(G369)

Figure 4 (cont.). Secondary Metabolites Isolated and Characterized (continued)

2.2.12 Berkeleyacetal C

Berkeleyacetal C was first isolated in 2007 from a deep was *Penicillium* sp. growing in Berkeley Pit Lake in Butte, Montana. It inhibited both MMP-3 and caspase-1 in the micromolar range and was later accepted in the NCI Developmental Therapeutics Program for human cell line screening and tested in the single-dose-response assay. It inhibited the growth of non-small-cell lung cancer NCI H460.[28] Berkeleyacetal C was isolated from an unidentified freshwater fungus (G173). It was tested in anti-cancer, anti-bacterial, and anti-amoeba bioassays; however, it was found to be inactive in all of them.

2.2.13 Madisone

Madisone was first isolated in 2015 from *Lindgomyces madisonensis* which was isolated from a sample collected in a stream in Madison, North Carolina. Madisone was tested against *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium smegmatis*, *Candida albicans*, and *Aspergillus niger* and has minimal inhibitory concentrations greater than 55 μg/mL in all bioassays.[29] Madisone was isolated from *Lindgomyces madisonensis* (Lindgomycetaceae) (G416). It was tested in a variety of bioassays and found to be inactive in all of them.

2.2.14 4'-Methoxymadisone

4'-Methoxymadisone was first isolated as a natural product in 2015 from *Lindgomyces madisonensis* which was isolated from a sample collected in a stream in Madison, North Carolina. It was tested against *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium smegmatis*, *Candida albicans*, and *Aspergillus niger* and had minimal inhibitory concentrations greater than 55 μg/mL in all bioassays.[29] 4'-Methoxymadisone

was isolated from *Lindgomyces madisonensis* (Lindgomycetaceae) (G416) and characterized as inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

2.2.15 Dimadisone

Dimadisone was first isolated as a natural product in 2015 from *Lindgomyces madisonensis* which was isolated from a sample collected in a stream in Madison, North Carolina. It was not evaluated for antimicrobial activity due to the relative lack of material.[29] Dimadisone was isolated from *Lindgomyces madisonensis* (Lindgomycetaceae) (G416) and determined to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

2.2.16 Monascuspyrone

Monascuspyrone was first isolated in 2010 in Taiwan from an extract of red mold rice fermented with the yellow mutant of the fungus *Monascus pilosus*.[30] Monascuspyrone was isolated from *Microascus nidicola* (Microascaceae) (G377) and tested in various biological assays. It was inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays; however, it was partially active against *Staphylococcus aureus* with 52.7% (±1.3) inhibition at 100 μg/mL and 20.4% (±2.6) inhibition at 10 μg/mL (Figure 5).

2.2.17 Monascuskaoliaone

Monascuskaoliaone was first isolated in 2010 in Taiwan from an extract of *Monascus kaoliang*.[31] was isolated from *Microascus nidicola* (Microascaceae) (G377) and tested in various biological assays where it was determined to be inactive against cancer, bacteria, and amoeba.

2.2.18 Greensporone A

Greensporone A was first isolated in 2014 from a freshwater aquatic fungus *Halenospora* sp. originating from a stream in Greensboro, North Carolina. It was tested against melanoma and colon cancer cell lines yielding IC_{50} values of 14.1 μM and greater than 20 μM, respectively.[32] Greensporone A was re-isolated from the same fungus (G87) as the original isolation. When tested in anti-cancer, anti-bacterial, and anti-amoeba bioassays, it was found to be inactive.

2.2.19 Greensporone B

Greensporone B was first isolated in 2014 from a freshwater aquatic fungus *Halenospora* sp. originating from a stream in Greensboro, North Carolina. It was tested against melanoma and colon cancer cell lines and found to be inactive against both.[32] Greensporone B was re-isolated from the same fungus (G87) as the original isolation. When tested in anti-cancer, anti-bacterial, and anti-amoeba bioassays, it was found to be inactive.

2.2.20 8,9-Dihydrogreensporone A

8,9-Dihydrogreensporone A was first isolated in 2014 from a freshwater aquatic fungus *Halenospora* sp. originating from a stream in Greensboro, North Carolina. It was tested against melanoma and colon cancer cell lines and found to be inactive against both.[32] 8,9-Dihydrogreensporone A was re-isolated from the same fungus (G87) as the original isolation. When tested in anti-cancer, anti-bacterial, and anti-amoeba bioassays, it was found to be inactive.

2.2.21 Dehydroisopenicillide

Dehydroisopenicillide was first isolated in 1991 in Japan. Dehydroisopenicillide was isolated from a Leotiomycetes sp. (G369) and characterized via HRMS and ^{1}H NMR in reference to the published values.[33] When tested in a variety of bioassays, it was found to be inactive.

Table 4. ^{1}H (400 MHz) NMR Data for Dehydroisopenicillide in $CDCl_3$

Position	Literature Values δ_H (J, Hz)	Observed δ_H (J, Hz)
4’ , 5’	1.44 (6H, *s*)	1.43 (6H, *s*)
3’ –OH	1.58 (1H, *br s*)	
9 –Me	2.25 (3H, *s*)	2.23 (3H, *s*)
4 – OMe	3.93 (3H, *s*)	3.92 (3H, *s*)
7	5.07 (2H, *br s*)	5.06 (2H, *br s*)
11 –OH	6.09 (1H, *br s*)	5.96 (1H, *br s*)
2’	6.38 (1H, *d*, 15.6)	6.38 (1H, *d*, 16.2)
8	6.38 (1H, *d*, 1.8)	6.38 (1H, *d*, 1.0)
1’	6.81 (1H, *d*, 15.6)	6.80 (1H, *d*, 16.3)
10	6.85 (1H, *d*, 1.8)	6.85 (1H, *d*, 1.7)
1	6.86 (1H, *d*, 8.6)	6.86 (1H, *d*, 8.5)
2	7.56 (1H, *d*, 8.6)	7.57 (1H, *d*, 8.5)

2.2.22 Penicillide

Penicillide was first isolated in 1974 in Japan from a *Penicillium* sp.[34] Penicillide was isolated from a Leotiomycetes sp. (G369) and found to be inactive in anti-cancer, anti-bacterial, and anti-amoeba bioassays.

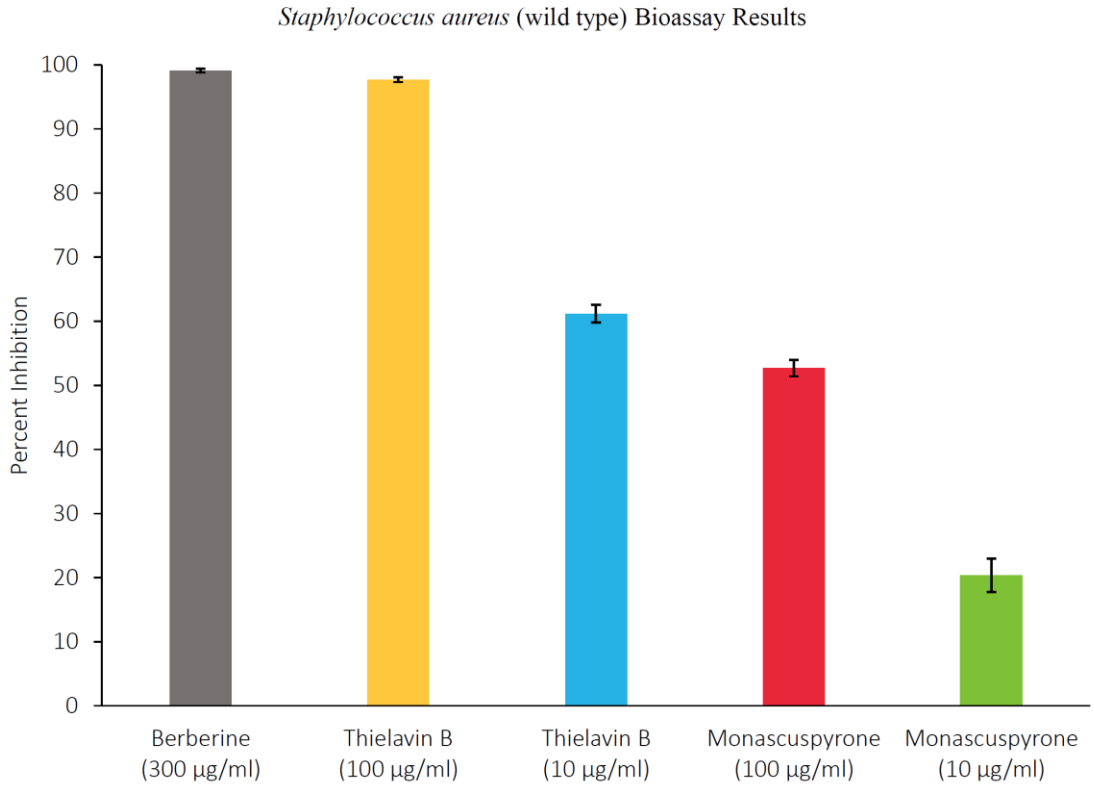


Figure 5. Percent Inhibition of *Staphylococcus aureus* at Various Concentrations of Various Compounds

2.3 Conclusion

Maintaining supply of secondary metabolites is an ongoing challenge in natural products research. There are two options for approaching this challenge: isolate a plethora of compound early on or re-isolate later on. The former approach would require a researcher to isolate large amounts of compound and the ability to store the material where it will not degrade. This storage would allow for further experiments to be done on these metabolites later on, such as, semi-synthesis or various biological assays. The risk of this approach is degradation of the metabolites while in storage. The latter approach would require storage of the fungal culture and regrowth later on. This would take advantage of the biological variance of fungi, and this would allow for secondary metabolites that were not previously characterized from these fungi to be obtained and analyzed. The risk of this approach is fungi not surviving. This project helped show that both approaches would yield successful yet different results.

CHAPTER III

PRODUCTION OF GRISEOFULVIN AND DECHLOROGRISEOFULVIN BY *XYLARIA* SPP.

3.1 Experimental

3.1.1 Isolation of Fungal Cultures

Xylaria flabelliformis (strain G536; previously named *Xylaria cubensis*) was isolated as an endophyte from surface sterilized twigs of paw paw (*A. triloba* (L.) Dunal, Annonaceae) collected from Pfafftown, NC, USA (36º09'58.8"N 80º24'18.6"W) and its draft genome was reported recently.[12, 35] The additional fungal cultures studied, along with G536, are preserved at the University of North Carolina at Greensboro, Department of Chemistry and Biochemistry Fungal Culture Collection.

3.1.2 Cultures in Petri Plate

The fungi were maintained on potato dextrose agar (PDA; Difco) containing antibiotics. To establish the individual monocultures, an agar plug from the leading edge of the colony was cut out aseptically and transferred onto Petri plates with PDA containing antibiotics. Twelve Petri plates of each fungal strain, 36 plates in total, were prepared. The Petri plates were incubated at room temperature (~22ºC). At each time point, three biological replicates of each strain were analyzed.

3.1.3 Extraction of Fungal Cultures

The fungal growth was ceased by spraying the culture with methanol. The contents of the Petri plate were then sliced and transferred into a scintillation vial. The vial was then filled with acetone, capped, and left alone for four hours. The solution was removed from the vial using a pipette and transferred to a clean, weighed vial and left to dry under nitrogen. The dry extract was then treated with a 1:1 ethyl acetate:water mixture in order to remove any remaining agar solution. The organic layer was then collected and dried. The organic extract was used for the chemical analysis done in this experiment.

3.1.4 Preparation of Griseofulvin and Dechlorogriseofulvin Standards

Aliquots of each metabolite were weighed and prepared with methanol at a concentration of 1.05 mg/mL, then 0.50 mL of each solution were combined in a 1.5 mL Eppendorf tube to yield a solution containing 0.525 mg/mL of each compound. Serial dilutions were conducted to produce 20 concentrations.

3.1.5 Preparation of Extracts for LC-MS

The organic extracts were prepared at a 5 μg/mL concentration, then 200 μL of the solution was transferred to an Ansi 96 well – 1 mL plate.

3.1.6 LC-MS Analysis

To quantify griseofulvin and dechlorogriseofulvin production, LC-MS analysis was conducted in positive ion mode. The mass spectrometer scanned across a mass range of *m/z* 250 to 450 at a resolution of 70,000, and a spray voltage of 4,000. It was coupled to an Acquity UPLC system (Waters Corp.), which had a flow rate of 0.3 mL/min and

utilized a BEH C_{18} column (2.1 mm x 50 mm, 1.7 μm) that was operated at 40°C. The mobile phase consisted of Fisher Optima LC-MS grade CH_3CN-H_2O (both acidified with 0.1% formic acid). The gradient began at 15% CH_3CN and linearly increased to 100% CH_3CN over 8 min. It was held at 100% for 1.5 min before returning to starting conditions to re-equilibrate. Each sample was ran in triplicate. The data was analyzed using Thermo Xcalibur Qual Browser and Thermo Xcalibur Quan Browser. The quantitation curves for griseofulvin and dechlorogriseofulvin were prepared with a quantitation range of 1 ng/mL to 4096 ng/mL and 1 ng/mL to 256 ng/mL, respectively.

3.2 Results

Morphological results revealed that G1079 grows the fastest while G755 grows the slowest. By week four, the bottom of the plates reveals dark rings beginning to form which are common in *Xylaria* sp. (Figure 6).

Figure 6. Pictures of *Xylaria* spp. Growth Over Four Weeks The first four columns display a view of the top of the plate. The last column displays the bottom of a plate that had been growing for four weeks.

The *Xylaria* strains worked with mainly produce two compounds; griseofulvin and dechlorogriseofulvin. Griseofulvin is known for its fungistatic properties. The metabolite production of each fungal strain at each time point was measured using calibration curve. The calibration curves used to measure griseofulvin production (Figure 7) and dechlorogriseofulvin production (Figure 8) were created using standards and ran in triplicate.

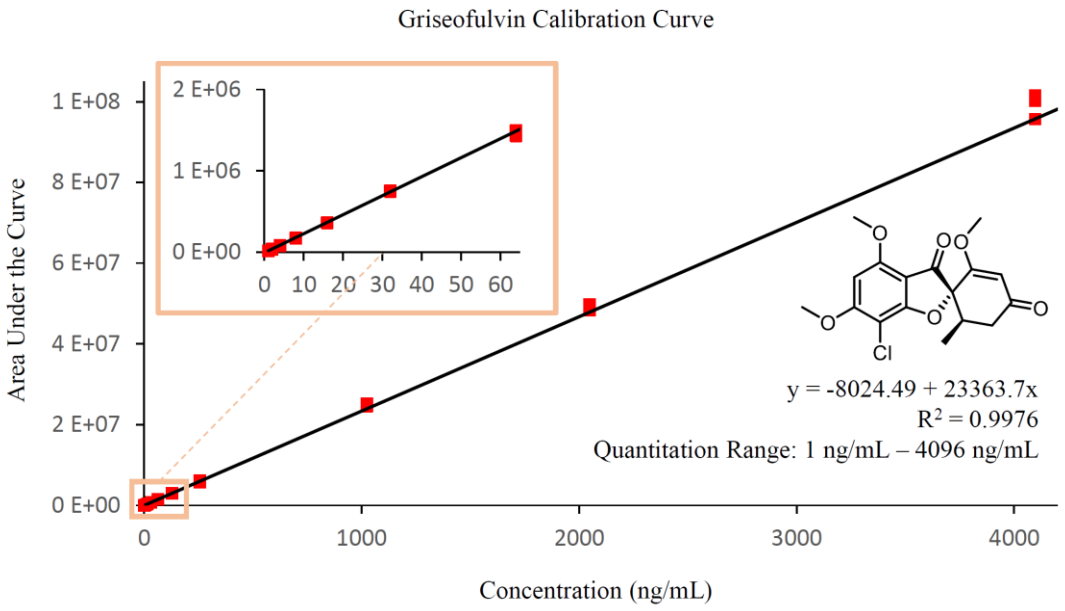


Figure 7. Calibration Curve Used to Measure the Amount of Griseofulvin Present in Extracts

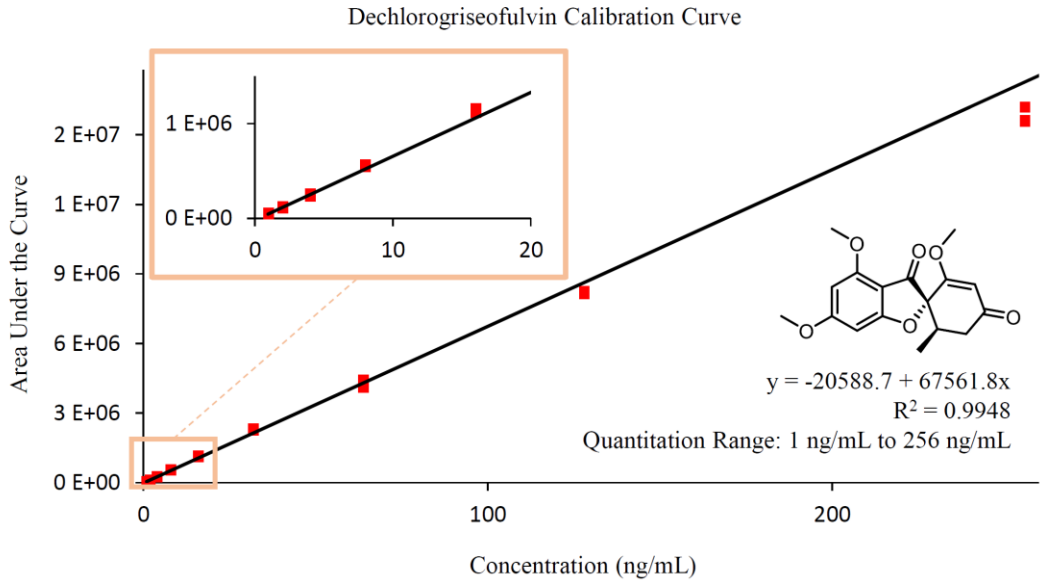


Figure 8. Calibration Curve Used to Measure the Amount of Dechlorogriseofulvin Present in Extracts

G1079 successfully produced griseofulvin and dechlorogriseofulvin; however, the amount produced of both metabolites did not vary over the period of four weeks (Figures 9 and 10). The griseofulvin production by G536 at week two was not significantly different in comparison to the first week, however, week three did show a significant difference when compared to week one. Week four is not significantly different than week three, which implies that G536 is reaching a maximum production of griseofulvin within three weeks of growth (Figure 9). This metabolic trend is not seen when analyzing dechlorogriseofulvin production by G536. A significant difference in dechlorogriseofulvin production is seen when comparing weeks one and two; however, there is no significant difference after week two. Indicating that dechlorogriseofulvin production is reaching its maximum within two weeks of growth for G536 (Figure 10). G755 reaches its maximum griseofulvin production after four weeks of growth because there is a significant difference when comparing weeks one and four. Conversely, there is no significant difference amongst the first three weeks (Figure 9). Dechlorogriseofulvin biosynthesis in G755 does not follow the same trend, rather there is no significant difference over the course of four weeks implying that it reaches its maximum production by week one (Figure 10). When comparing the three *Xylaria* spp. (G1079, G536, G755), G536 and G755 produce significantly more griseofulvin and dechlorogriseofulvin than G1079 within four weeks of growth. The production of griseofulvin by G536 and G755 is not significantly different at their maxima; however, G536 produces a greater abundance of dechlorogriseofulvin than G755 (Figure 10). G536 has the highest rate of griseofulvin production, whereas G1079 has the lowest production rate. G536 produces

dechlorogriseofulvin at a faster rate than both G755 and G1079, which have similar rates of production.

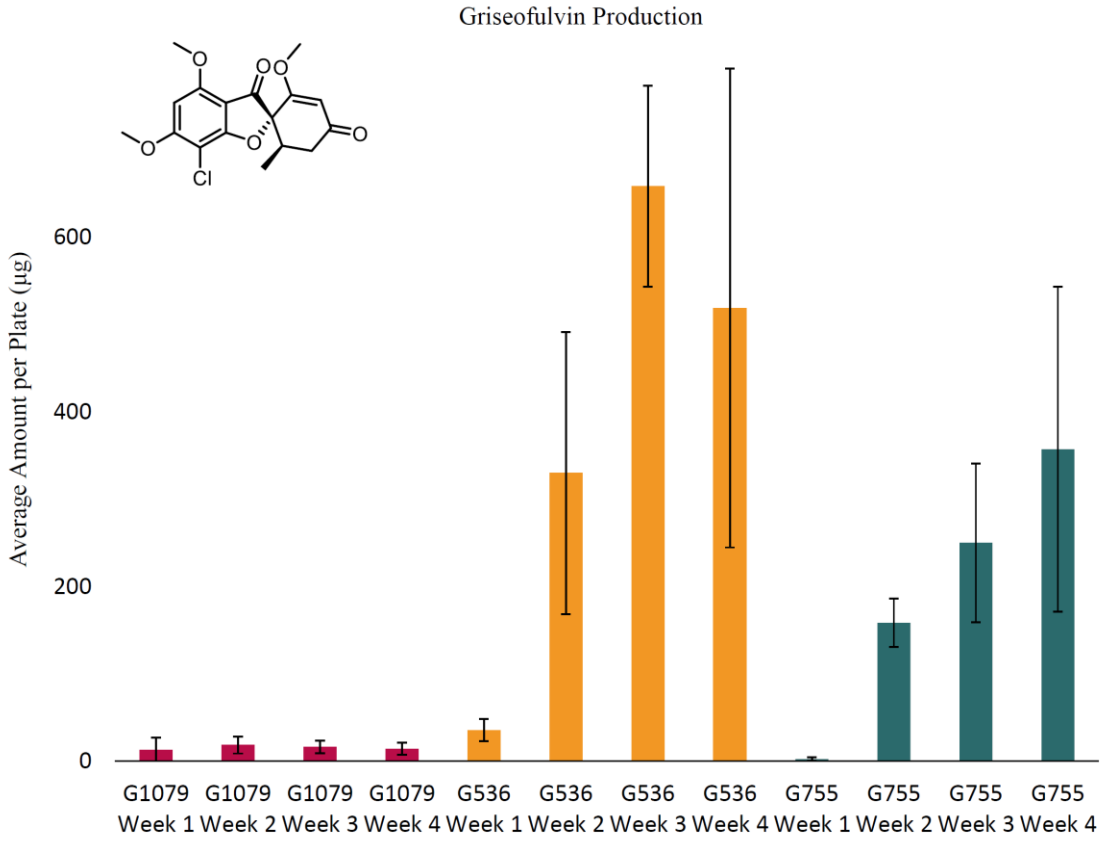


Figure 9. Griseofulvin Production Comparison of the Three *Xylaria* spp. Strains Over Four Weeks

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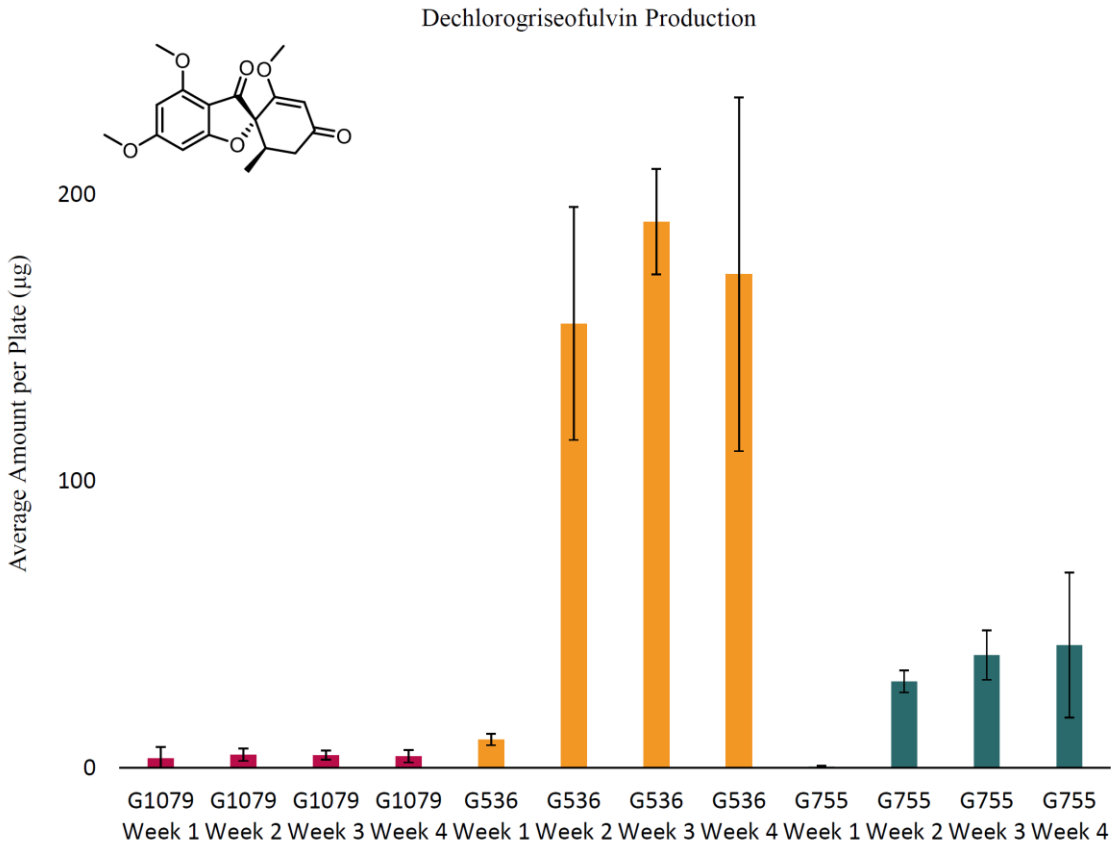

Figure 10. Dechlorogriseofulvin Production Comparison of the Three *Xylaria* spp. Strains Over Four Weeks

A co-culture of *Xylaria flabelliformis* (G536) and fungal strain G959 was analyzed in comparison to the monocultures of each strain. The chromatographic data collected on the mass spectrometer indicated the presence of a compound that is not present in either of the monocultures. To continue this study, the co-culture will need to be scaled-up on solid media. The goal will be to isolate and characterize the compound which is not present in either of the monocultures.

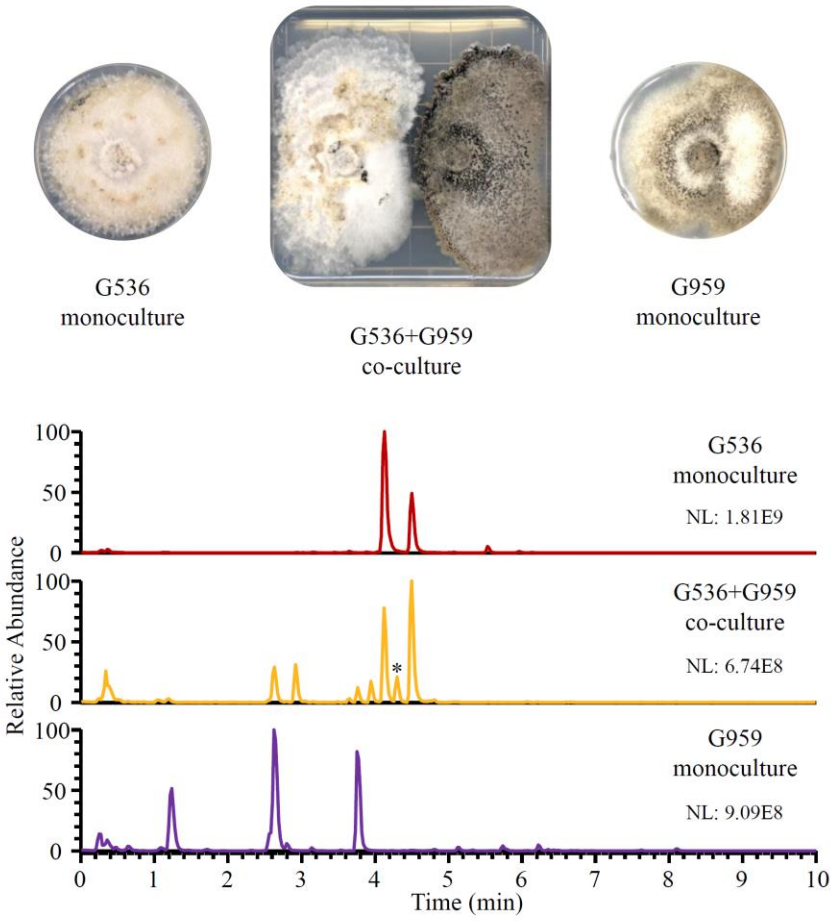

Figure 11. Monocultures of *Xylaria flabelliformis* (G536) and G959. Co-Culture of G536 and G959. Full Base Peak Chromatograms of all of the Cultures.

3.3 Conclusion

These results suggest that G536 is the optimal *Xylaria* sp. for co-culturing experiments since it biosynthesizes more griseofulvin thus creating a more competitive environment. Alternatively, G755 may also prove useful in co-culturing experiments since it produces similar amounts of griseofulvin but at a slower rate. Allowing for longer growth times when needed. There are many fungal strains that can be used in opposition of *Xylaria* sp. in hopes of activating silent biosynthetic gene clusters thus diversifying secondary metabolite production. Further analysis of more *Xylaria* spp. could provide the necessary data to optimize co-culturing experimental design.

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